Therefore, adenovirus designated CV 834 comprises probasin promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B, a deletion of the E1B endogenous promoter and a deleted E3 region.

Example 9: Construction of a Replication-Competent Adenovirus Vector with a hCMV-TRE and an EMCV IRES

The hCMV immediate early gene (IE) promoter from plasmid CP629, originally derived from pCMVBeta (Clonetech, Palo Alto) was inserted at the PinAI site of plasmid CP627 (see Example 8) to generate CP629, containing a CMV IE promoter upstream of E1A and an IRES between E1A and E1B. Full-length viral genomes were obtained by recombination between CP629 and a plasmid containing a right arm of an adenovirus genome. The right arms used in virus recombination were pBHGE3, containing an intact E3 region, and pBHG11 or pBHG10 containing a deletion in the E3 region. The structure of all viral genomes was confirmed by conducting PCR amplifications that were diagnostic for the corresponding specific regions.

Therefore, adenovirus vector designated CV835 comprises hCMV-IE promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B a deletion in the E1B endogenous promoter and a deleted E3 region. CV835 lacks the hCMV enhancer and is therefore not tissue specific. By adding the hCMV IE enhancer sequence to CV835, the vector is made tissue specific.

Example 10: Comparison of Dual TRE Vectors with Single TRE/IRES-Containing Vectors

Two liver cancer-specific adenovirus vectors, CV790 and CV733 (also designated CN790 and CN733, respectively), were generated and characterized. See PCT/US98/04084. These viruses contain two AFP TREs, one upstream of E1A and one upstream of E1B. They differ in that CV790 contains an intact E3 region, while the E3 region is deleted in CV733. Replication of these two viruses was compared with that of the newly generated IRES-containing viruses, CV890 and CV840 (*see* Example 1).

Virus replication was compared, in different cell types, using a virus yield assay as described in Example 4. Cells were infected with each type of virus and, 72 hrs after infection, virus yield was determined by a plaque assay. The results indicate that vectors containing an IRES between E1A and E1B (CV890 and CV840), in which E1B translation is regulated by the IRES, replicate to similar extents as normal adenovirus and viruses with dual AFP TREs, in AFP-producing cells such as 293 cells and hepatoma cells. In SK-Hep-1 (liver cells), PA-1 (ovarian carcinoma) and LNCaP cells (prostate cells) the IRES-containing viruses do not replicate as well as dual TRE or wild-type adenoviruses, indicating that the IRES-containing viruses have higher specificity for hepatoma cells. Based on these results, it is concluded that IRES-containing vectors have unaltered replication levels, but are more stable and have better target cell specificity, compared to dual-TRE vectors.

Example 11: Uroplakin adenoviral constructs containing an EMCV IRES

A number of E3-containing viral constructs were prepared which contained uroplakin II sequences (mouse and/or human) as well as an EMCV internal ribosome entry site (IRES). The viral constructs are summarized in Table 11. All of these vectors lacked an E1A promoter and retained the E1A enhancer.

The 519 base pair EMCV IRES segment was PCR amplified from Novagen's pCITE vector by primers A/B:

primer A: 5'-GACGTCGACTAATTCCGGTTATTTTCCA

primer B 5'-GACGTCGACATCGTGTTTTTCAAAGGAA (*GTCGAC* is a SalI site).

The EMCV IRES element was ligated to PCR blunt vector (Invitrogen pCR® blunt vector).

CP1066

The 1.9kb-(-1885 to +1) fragment of mouse UPII from CP620 was digested with AflIII (blunted) and HindIII and inserted into pGL3-Basic from CP620 which had been digested with XhoI (blunted) and HindIII to generate CP1066.

CP1086

The 1.9kb mouse UPII insert was digested with PinAI and ligated with CP269 (CMV driving E1A and IRES driving E1B with the deletions of E1A/E1B endogenous promoter) which was similarly cut by PinAI.

CP1087

The 1kb (-1128 to +1) human UPII was digested with PinAI from CP665 and inserted into CP629 which had been cut by PinAI and purified (to elute CMV).

CP1088

The 2.2kb (-2225 to +1) human UPII was amplified from CP657 with primer 127.2.1 (5'-AGGACCGGTCACTATAGGGCACGCGTGGT-3') PLUS 127.2.2 (5'-AGGACCGGTGGGATGCTGGGCTGGGAGGTGG-3') and digested with PinAI and ligated with CP629 cut with PinAI.

CP627 is an Ad5 plasmid with an internal ribosome entry site (IRES) from encephelomycarditis virus (EMCV) at the junction of E1A and E1B. First, CP306 (Yu et al., 1999) was amplified with primer pairs 96.74.3/96.74.6 and 96.74.4/96.74.5.

The two PCR products were mixed and amplified with primer pairs 96.74.3 and 96.74.5. The resultant PCR product contains a 100bp deletion in E1A-E1B intergenic region and a new SalI site at the junction. EMCV IRES fragment was amplified from pCITE-3a(+) (Novagen) using primers 96.74.1 and 96.74.2. The SalI fragment containing IRES was placed into SalI site to generate CP627 with the bicistronic E1A-IRES-E1B cassette. CP629 is a plasmid with CMV promoter amplified from pCMVbeta (Clontech) with primer 99.120.1 and 99.120.2 and cloned into PinAI site of CP627.

CP657 is a plasmid with 2.2kb 5' flanking region of human UP II gene in pGL3-Basic (Promega). The 2.2kb hUPII was amplified by PCR from

GenomeWalker product with primer 100.113.1 and 100.113.2 and TA-cloned into pGEM-T to generate CP655.

The 2.2kb insert digested from SacII (blunt-ended) and KpnI was cloned into pGL3-Basic at HindIII (blunted) and KpnI to create CP657.

CP1089

The 1kb (-965 to +1) mouse UPII was digested by PinAI from CP263 and inserted into CN422 (PSE driving E1A and GKE driving E1B with the deletions of E1A/E1B endogenous promoter) cut by PinAI and purified and further digested with EagI and ligated with 1kb (-1128 to +1) human UPII cut from CP669 with EagI.

CP1129

The 1.8kb hUPII fragment with PinAI site was amplified from CP657 with primer 127.50.1 and 127.2.2 and cloned into PinAI site of CP629.

CP1131

CP686 was constructed by replacing the CMV promoter in CP629 with an AFP fragment from CP219. A 1.4kb DNA fragment was released from CP686 by digesting it with BssHII, filling with Klenow, then digesting with BglII. This DNA fragment was then cloned into a similarly cut CP686 to generate CP1199. In CP1199, most of the E1B 19-KDa region was deleted. The 1.8kb hUPII fragment with PinAI site was amplified from CP657 by PCR with primer 127.50.1 and 127.2.2 and inserted into similarly digested CP1199 to create CP1131.

The plasmids above were all co-transfected with pBHGE3 to generate CV874 (from CP1086), CV875 (from CP1087), CV876 (from 1088) and CV877 (from CP1089), CV882 (from CP1129) and CV884 (from CP1131). CP1088, CP1129 and CP1131 were cotransfected with pBHGE3 for construction of CV876, CV892 and CV884, respectively by lipofectAMINE (Gibco/BRL) for 11-14 days. pBHGE3 was purchased from Microbix, Inc., and was described previously. The cells were lysed by three freeze-thaw cycles and plaqued on 293 cells for a week. The single plaques were picked and amplified by infection in 293 cells for 3-5 days. The viral DNAs were isolated from the lysates and the constructs were

confirmed by PCR with primer 31.166.1/ 51.176 for CV876 and primer 127.50.1/51.176 for CV882 and CV884 at E1 region and primer 32.32.1/2 for all three viruses at E3 region.

TABLE 11

Name	Vector	Ad 5 Vector	E1A TRE	E1B TRE	E3
CV874	CP1086	pBHGE3	1.9 kb mUPII	IRES	intact
CV875	CP1087	pBHGE3	1.0 kb hUPII	IRES	intact
CV876	CP1088	pBHGE3	2.2 kb hUPII	IRES	intact
CV877	CP1089	pBHGE3	1.0 kb mUPII	1.0 kb hUPII (E1B promoter deleted)	intact
CV882	CP1129	pBHGE3	1.8 kb hUPII	IRES	intact
CV884	CP1131	pBHGE3	1.8 kb hUPii	IRES (E1B 19-kDa deleted)	intact

Viruses are tested and characterized as described above.

Primer sequences:

96.74.1	GACGTCGACATCGTGTTTTTCAAAGGAA
96.74.2	GACGTCGACTAATTCCGGTTATTTTCCA
96.74.3	CCTGAGACGCCCGACATCACCTGTG
96.74.4	TGCTGAATGGTCGACATGGAGGCTTGGGAG
96.74.5	CACAACCGCTCTCCACAGATGCATG
96.74.6	GTCGACCATTCAGCAAACAAAGGCGTTAAC
100.113.1	AGGGGTACCCACTATAGGGCACGCGTGGT
100.113.2	ACCCAAGCTTGGGATGCTGGGCTGGGAGGTGG
127.2.2	AGGACCGGTGGGATGCTGGGCTGGGAGGTGG
127.50.1	AGGACCGGTCAGGCTTCACCCCAGACCCAC
31.166.1	TGCGCCGGTGTACACAGGAAGTGA
32.32.1	GAGTTTGTGCCATCGGTCTAC
32.32.2	AATCAATCCTTAGTCCTCCTG
51.176	GCAGAAAAATCTTCCAAACACTCCC
99.120.1	ACGTACACCGGTCGTTACATAACTTAC
99.120.2	CTAGCAACCGGTCGGTTCACTAAACG

Example 12: Construction of a Replication-Competent Adenovirus Vector with a Tyrosinase TRE and EMCV IRES

CP621 is a plasmid containing a human tyrosinase enhancer and promoter elements in a PinAl fragment. This fragment is ligated to the PinAl site on CP627 to generate CP1078. CP1078 is combined with pBHGE3 to generate a new melanoma specific virus, CV859. Table 14 depicts the polynucleotide sequence of the PinAl fragment which contains a tyrosinase promoter and enhancer.

Example 13: Construction of a Replication-Competent Adenovirus Vector with a Probasin-TRE and a VEGF IRES

Using a strategy similar to that described in Example 8, the IRES fragment from the mouse vascular endothelial growth factor (VEGF) gene is amplified and cloned into CP628 at the SalI site. Table 12 depicts the IRES fragment obtainable from vascular endothelial growth factor (VEGF) mRNA. In order to clone this fragment into the E1a/E1b intergenic region, two pieces of long oligonucleotide are synthesized. The sense oligonucleotide is shown in the Table , whereas the second piece is the corresponding antisense one. After annealing the two together to create a duplex, the duplex is subjected to SalI digestion and the resulting fragment is cloned into the SalI site on CP628. The resulting plasmid, CP630, has a probasin promoter in front of E1a and an VEGF IRES element in front of E1b. This plasmid is used to construct a prostate cancer-specific virus comprising the VEGF IRES element.

Example 14: Construction of a Replication-Competent Adenovirus Vector with an AFP-TRE and a VEGF IRES

Using a strategy similar to Example 8, a PinAI fragment which contains AFP TRE can be obtained. This AFP TRE is cloned into the PinA1 site in front of E1A on CP628 yielding plasmid CP1077. This plasmid has the AFP TRE for E1 transcriptional control and the VEGF IRES element before E1b. CP1077 can be recombined with pBHGE3 to generate a liver-specific adenovirus, designated as CV858.

Example 15: Construction of a Replication-Competent AdenovirusVector with a hKLK2-TRE and a EMCV IRES

Using a strategy similar to Example 1, the TRE fragment from human glandular kallikrein II as shown in Table 14 was cloned into the PinAI site in CP627. The resultant plasmid, CP1079, is cotransfected with pBHGE3 to create CV860.

Example 16: Construction of a Replication-Competent Adenovirus Vector with a CEA-TRE and a EMCV IRES

Using a strategy similar to Example 1, the TRE fragment from Carcinembryonic antigen (CEA)(Table 14, SEQ ID NO:__) is used to construct virus designated CV873. A PinAI fragment containing the CEA-TRE was cloned into the PinAI site in front of E1A of CP627 for the transcriptional control. The resultant plasmid CP1080 is used together with pBHGE3 to generate CV873.

Example 17: Adenovirus Vectors with Urothelial Cell-Specific TREs

A number of plasmid constructs were generated as intermediates for adenovirus type 5 (Ad 5) vector constructs. The plasmid constructs were based on plasmid CP321 (Yu et al., 1999, *Cancer Res.* 59:4200-4203), which contains a prostate-specific enhancer inserted at a PinAI site upstream of the E1A gene and at a EagI site upstream of the E1B gene. Constructs were created by inserting various UPII-derived 5'-flanking DNA sequences into the PinAI and EagI sites and removing the prostate-specific enhancer. Characteristics of the plasmid CP669 are E1A TRE 1.0kb hUPII and E1B TRE 1.0kb mUPI and lacked the E1A promoter and which contained the E1A enhancer. Infectious recombinant adenoviral vectors was produced by co-transfecting 293 cells with the UPII 5'-flanking DNA/E1 constructs and an Ad 5 backbone vector (pBHG10 or pBHGE3, Microbix, Inc.) as described in Yu et al. (*id.*) to produce CV829,which has an intact E3 region.

Example 18: *In vitro* Characterization of Melanocyte-Specific TRE-Containing Adenoviral Constructs

An especially useful objective in the development of melanocyte cell-specific adenoviral vectors is to treat patients with melanoma. Methods are

described below for measuring the activity of a melanocyte-specific TRE and thus for determining whether a given cell allows a melanocyte-specific TRE to function.

Cells and Culture Methods

Host cells such as, HepG2 (liver); Lovo (colon); LNCaP (prostate); PMEL (melanoma); SKMel (melanoma); G361 (melanoma) and MeWo cells are obtained at passage 9 from the American Type Culture Collection (Rockville, MD). MeWo cells are maintained in RPMI 1640 medium (RPMI) supplemented with 10% fetal bovine serum (FBS; Intergen Corp.), 100 units/mL of penicillin, and 100 units/mL streptomycin. MeWo cells being assayed for luciferase expression are maintained in 10% strip-serum (charcoal/dextran treated fetal bovine serum to remove T3, T4, and steroids; Gemini Bioproduct, Inc., Calabasas, CA) RPMI.

Transfections of MeWo Cells

For transfections, MeWo cells are plated out at a cell density of 5 x 10^5 cells per 6-cm culture dish (Falcon, NJ) in complete RPMI. DNAs are introduced into MeWo cells after being complexed with a 1:1 molar lipid mixture of N-[1-(2,3-dioleyloxy)propyl-*N,N,N*-trimethylammonium chloride (DOTAP™; Avanti Polar Lipids, AL) and dioleoyl-phosphatidylethanolamine (DOPE™; Avanti Polar Lipids, AL); DNA/lipid complexes are prepared in serum-free RPMI at a 2:1 molar ratio. Typically, 8 μg (24.2 nmole) of DNA is diluted into 200 μL of incomplete RPMI and added dropwise to 50 nmole of transfecting, lipids in 200 μL of RPMI with gentle vortexing to insure homogenous mixing of components. The DNA/lipid complexes are allowed to anneal at room temperature for 15 minutes prior to their addition to MeWo cells. Medium is removed from MeWo cells and replaced with 1 mL of serum-free RPMI followed by the dropwise addition of DNA/lipid complexes. Cells are incubated with complexes for 4-5 hours at 37°C, 5% CO_2. Medium was removed and cells washed once with PBS. The cells were then trypsinized and resuspended in 10% strip-serum RPMI (phenol red free). Cells were replated into an opaque 96-well tissue culture plate (Falcon, NJ) at a cell density of 40,000 cells/well per 100 μL media and assayed.

Plaque assays

To determine whether the adenoviral constructs described above replicate preferentially in melanocytes, plaque assays are performed. Plaquing efficiency is evaluated in the following cell types: melanoma cells (MeWo), prostate tumor cell lines (LNCaP), breast normal cell line (HBL-100), ovarian tumor cell line (OVCAR-3, SK-OV-3), and human embryonic kidney cells (293). 293 cells serve as a positive control for plaquing efficiency, since this cell line expresses Ad5 E1A and E1B proteins. For analyzing constructs comprising a melanocyte-specific TRE, cells that allow a melanocyte-specific TRE to function, such as the cell lines provided above and cells that do not allow such function, such as HuH7, HeLa, PA-1, or G361, are used. The plaque assay is performed as follows: Confluent cell monolayers are seeded in 6-well dishes eighteen hours before infection. The monolayers are infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the medium is removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques are scored two weeks after infection.

Example 19: *In vitro* and *In vivo* assays of anti-tumor activity

An especially useful objective in the development of urothelial cell-specific adenoviral vectors is to treat patients with bladder cancer. An initial indicator of the feasibility is to test the vector(s) for cytotoxic activity against cell lines and tumor xenografts grown subcutaneously in Balb/c nu/nu mice.

***In vitro* characterization of CV876**

Virus yield assay for CV876

5 X $10^5$293, RT-4, SW780, PA-1, G361, MKNI, HBL-100, Fibroblast (from lung) and Smooth muscle cells (from bladder) were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Unlike wt. Ad5, CV802 which grows well in all of the cells tested, CV876 replicates much better in permissive cells (293, RT-4 and SW780) than in non-permissive cells (PA-1, G361, MKN1, HBL-100 and primary cells) by about 100-10000 fold. Noticeably, the replication in SW780 for CV876 is about 100 fold less than CV802, which indicates the limitation of this virus in efficacy.

Growth curve experiment for CV876

5 X 10^5 RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with 133) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV876 replicates well only in RT-4 but not in primary cells and PA-1 over a 120h period of time. However, CV876 does show a delay of replication in RT-4 compared to CV802.

Cytopathic effect assay for CV876

5 X $10^5$293, RT-4, SW780, PA-1, MKN1 and LNCap were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV876 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (PA-1, MKN-1 and LNCap).

MTT assay for CV876

2 X $10^4$293, RT-4, SW780, MKN1, PA-1, HBL-100, Smooth muscle cells (from bladder) and Fibroblast (from lung) were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV876 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time point of day 1, 3,5,7 and 10. The medium was replaced by 50ul of MTT at 1mg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV876 shows efficacy only in permissive cells but not in non-permissive cells. Again, in RT-4 and SW780, CV876 kills the cells much slower than CV802.

***In vitro* characterization of CV882**

Virus yield assay for CV882

5 X $10^5$293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV882 in permissive cells (293, RT-4 and SW780) is comparable to CV802 (the difference is less than 100 fold) while it shows over 1000-1000000 fold difference in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells).

Growth curve experiment for CV882

5 X 10^5RT-4, PA-1, and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three, freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV882 replicates well only in RT-4 but not in primary cells and PA-1 over a 120h period of time. Additionally, CV882 shows better replication in RT-4 compared to CV876.

Cytopathic effect assay for CV882

5 X $10^5$293, RT-4, SW780, HBL-1 00, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPNI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV882 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (HBL-100, G361, PA-1 and Fibroblast cells).

MTT assay for CV882

2 X 10^4RT-4, SW780, PA-1, HBL-100, U118 and Fibroblast were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV882 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10. The medium was replaced by 50ul of MTT at lmg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C,

the solution was replaced by isopropanol and the plates were incubated at 30°C for lh and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV882 shows efficacy only in permissive cells but not in non-permissive cells.

In Vitro Characterization of CV884

Virus yield assay for CV884

5 X $10^5$293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV984 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV884 is very similar as CV802 in permissive cells (293, RT-4 and SW780) but shows over 1000 fold difference with CV802 in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells). CV884 shows better efficacy than CV876 and CV882 without losing much specificity.

Growth curve experiment for CV884

5 X 10^5RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV884 replicates very well only in RT-4 (similar as CV802) but not in primary cells and PA-1. Again, the replication of CV884 is better than CV882 and CV876.

Cytopathic effect assay for CV884

5 X $10^5$293, RT-4, SW780, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV884 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (G361, PA-I and Fibroblast cells).

MTT assay for CV884

2 X $10^4$293, RT-4, SW780, U118, Fibroblast and Smooth muscle cells were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV884 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10. The medium was replaced by 50ul of MTT at lmg/ml solution which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for lh and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV884 shows strong efficacy (similar as wt. Ad5) only in permissive cells but not in non-permissive cells.

In vivo activity of CV808

Mice were given subcutaneous (SC) injections of 1 x 10^6 sW780 cells. When tumors grew to about 500 mm^3, CV808 was introduced into the mice (5 X 10^7 PFU of virus in 0.1 ml PBS and 10% glycerol) intratumorally. Control mice received

vehicle alone. Tumor sizes were measured weekly. The data indicate that CV808 was effective at suppressing tumor growth.

While it is highly possible that a therapeutic based on the viruses described here would be given intralesionally (i.e., direct injection), it would also be desirable to determine if intravenous (IV) administration of adenovirus vector can affect tumor growth. If so, then it is conceivable that the virus could be used to treat metastatic tumor deposits inaccessible to direct injection. For this experiment, groups of mice bearing bladder epithelial tumors are inoculated with 10^8 to 10^{10} PFU of an adenoviral vector by tail vein injection, or with buffer used to carry the virus as a negative control. The effect of IV injection of the adenoviral vector on tumor size is compared to vehicle treatment.

Example 20: Synergistic Effect of CV 890 with Chemotherapeutics

Materials and Methods

Cells

Hepatocellular carcinoma cell lines HepG2, Hep3B, PLC/PRF/5, SNU449, and Sk-Hep-1, Chang liver cell (human normal liver cells), as well as other tumor cell lines PA-1 (ovarian carcinoma), UM-UC-3 (bladder carcinoma), SW 780 (bladder carcinoma), HBL100 (breast epithelia), Colo 201 (Colon adenocarcinoma), U 118 MG (glioblastoma) and LNCaP (prostate carcinoma) were obtained from the American Type Culture Collection. HuH-7 (liver carcinoma) was a generous gift of Dr. Patricia Marion (Stanford University). 293 cells (human embryonic kidney containing the E1 region of Adenovirus) were purchased from Microbix, Inc. (Toronto, Canada). The primary cells nBdSMC (normal human bladder smooth muscle cells), nHLFC (normal human lung fibroblast cells), and nHMEC (normal human mammary epithelial cells) were purchased from Clonetics (San Diego, California). All tumor cell lines were maintained in RPMI 1640 (BioWhittaker, Inc.) supplemented with 10% fetal bovine serum (Irvine Scientific), 100 U/ml penicillin and 100 ug/ml streptomycin. Primary cells were maintained in accordance with vendor instructions (Clonetics, San Diego). Cells were tested for the expression of AFP by immunoassay (Genzyme Diagnostics, San Carlos, CA).

Virus yield and one-step growth curves

Six well dishes (Falcon) were seeded with $5x10^5$ cells per well of calls of interest 24 hrs prior to infection. Cells were infected at an multiplicity of infection (MOI) of 2 PFU/cell for three hours in serum-free media. After 3 hours, the virus containing media was removed, monolayers were washed three times with PBS, and 4 ml of complete media (RPMI1640 + 10% FBS) was added to each well. 72 hours post infection, cells were scraped into the culture medium and lysed by three cycles of freeze-thaw.

The one-step growth curves time points were harvested at various time points after infection. Two independent infections of each virus cell-combination were titered in duplicate on 293 cells (Yu et al., 1999, *Cancer Research*, 59:1498-1504.

Northern blot analysis

Hep3B or HBL100 cells were infected at an MOI of 20 PFU/cell (plaque forming unit per cell) with either CV802 or CV890 and harvested 24 hours post infection. Total cell RNA was purified using the RNeasy protocol (Qiagen). The Northern blot was conducted using NorthernMax Plus reagents (Ambion, Austin, Texas). 5ug of RNA was fractionated on a 1% agarose, formaldehyde-based denaturing gel and transferred to a BrightStar-Plus (Ambion) positively charged membrane by capillary transfer. The antisense RNA probes for E1A (adenovirus genome 501bp to 1141bp) or E1B (1540bp-3910bp) were PCR products cloned in pGEM-T easy (Promega) and transcription labeled with [α ^{32}P] UTP. Blots were hybridized at 68°C for 14 hours with ZipHyb solution and washed using standard methods (Ambion). Membranes were exposed to BioMax film (Kodak).

Western blot analysis

Hep3B or HBL100 cells were infected at MOI of 20 PFU/cell with either CV802 or CV890 and harvested 24 hours post infection. Cells were washed with cold PBS and lysed for 30 min on ice in (50mM Tris, pH8.0, 150 mM NaCl, 1% IGEPAL CA360 a NP40 equivalent (Sigma), 0.5% sodium deoxycholate, and protease inhibitor cocktail from (Roche, Palo Alto, California). After 30 min centrifugation at 4C, the supernatant was harvested and the protein concentration determined with protein assay ESL kit (Roche). Fifty micrograms of protein per lane were separated on 816% SDS-PAGE and electroblotted onto Hybond ECL membrane (Amersham Pharmacia, Piscataway, New Jersey). The membrane was

blocked overnight in PBST (PBS with 0.1% Tween-20) supplemented with 5% nonfat dry milk. Primary antibody incubation was done at room temperature for 2-3 hrs with PBST/1 % milk diluted antibody, followed by wash and 1 hr incubation with diluted horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California). Enhanced chemiluminescence (ECL; Amersham Pharmacia) was used for the detection. E1A antibody (clone M58) was from NeoMarkers (Fremont, California), E1B-21 kD antibody was from Oncogene (Cambridge, Massachusetts). All antibodies were used according manufacturer's instruction.

Cell viability assay and statistical analysis

To determine the cell killing effect of virus and chemotherapeutic agent in combination treatment, a cell viability assay was conducted as previously described with modifications (Denizot, 1986, Journal Immunology. Methods, 89:271-277). On 96 well plates, cells of interest were seeded at 10,000 calls per well 48 hr prior to infection. Cells were then treated with virus alone, drug alone, or in combination. Cell viability was measured at different time points by removing the media, adding 50 µl of 1mg/ml solution of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-^{2}H-tetrazolium bromide) (Sigma, St. Louis, MO) and incubating for 3 hrs at 37°C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 50 µl of isopropanol followed by 30C incubation for 0.5 hr. The absorbency was determined on a microplate reader (Molecular Dynamics) at 560 nm (test wavelength) and 690 nm (reference wavelength). The percentage of surviving cells was estimated by dividing the OD_{550}-OD_{650} of virus or drug treated cells by the OD_{550}-OD_{650} of control cells. 6 replica samples were taken for each time point and each experiment was repeated at least three times.

For statistical analysis, CurveExpert (shareware by Daniel Hyams, version 1.34) was used to plot the dose-response curves for virus and drugs. Based upon the dose-response curves, the isobolograms were made according to the original theory of Steel and Peckham (1993, *Int. J. Rad. Onc. Biol. Phys.*, 5:85) and method described in Aoe et al. (1999, *Anticancer Res.* 19:291-299).

Animal studies

Six to eight week old athymic BALB/C *nu/nu* mice were obtained from Simonson Laboratories (Gilroy, California) and acclimated to laboratory conditions

one week prior to tumor implantation. Xenografts were established by injecting $1x10^6$ Hep3B, HepG2 or LNCaP cells suspended in 100µl of RPMI 1640 media subcutaneously. When tumors reached between 200 mm^3 and 300 mm^3, mice were randomized and dosed with 100 µl of test article via intratumoral or the tail vein injection. Tumors were measured in two dimensions by external caliper and volume was estimated by the formula [length (mm) x width $(mm)^2$]/2. Animals were humanely killed when their tumor burden became excessive. Serum was harvested weekly by retro-orbital bleed. The level of AFP in the serum was determined by AFP Immunoassay kit (Genzyme Diagnostics, San Carlos, CA). The difference in mean tumor volume and mean serum AFP concentration between treatment groups was compared for statistical significance using the unpaired, two-tailed, *t*-test.

Transcription and Translation of E1A/E1B Bicistronic Cassette of CV890 in Different Cells

In wild type adenovirus infection, E1A and E1B genes produce a family of alternatively spliced products. It has been found that there are five E1A mRNAs, among them 12S (880 nucleotides, nts) and 13S (1018 nts) mRNAs are the dominant ones that are expressed both early and late after infection. The 12S and 13S mRNAs encode the gene product of 243 amino acids (243R) and 289 amino acids (289R) respectively (reviewed by Shenk, 1996). The two major E1B transcripts that code for 19kD and 55kD proteins are 12S (1031 nts) and 22S (2287 nts) mRNAs. E1B 12S mRNA only codes the 19kD product, whereas the 22S mRNA codes for both 19kD and 55kD products due to different initiation sites during translation. In the current study, the generation of E1A-IRES-E1B bicistronic cassette was expected to change the pattern of E1A and E1B transcripts in viral infection. Therefore, Northern blot analysis was conducted to evaluate the steady-state level of E1A and E1B transcripts. First, CV802 or CV890 were infected to Hep3B (AFP) or HBL100 (AFP) cells for 24 hours. The total RNA samples were separated on agarose gels and processed for Northern blot by hybridizing to antisense RNA probes. The Northern blot with E1A probe visualized the 12S and 13S mRNAs in both wild type CV802 infected cells. For CV890, E1A transcripts can only be seen in Hep3B cells, indicating the conditional transcription of E1A. It is of interest to find that in CV890, there is only one large transcript (about 3.51Kb), whereas the 12S and 13S mRNAs are no longer present. This large transcript indicates the continuous transcription of E1A-IRES-E1B bicistronic cassette, suggesting an alteration of viral E1A splicing

pattern in CV890. Transcription of E1B from CV890 also appears to be AFP-dependent. It is clear that both 12S and 22S mRNAs of E1B were present in wild type CV802 samples, whereas the 128 mRNA and an enlarged 22S mRNA (3.5Kb) appeared in CV890 infected cells. Obviously, the identity of this enlarged mRNA is the same 3.5Kb transcript as visualized in E1A blot, which is from the transcription of E1A/E1B bicistronic cassette. Therefore, the E1B mRNA is tagged after E1A mRNA in this large transcript. This large transcript contains all the coding information for E1A, E1B 19kD and E1B 55kD. The mRNA splice pattern that appears in CV802 is not valid in CV890, the12S mRNA with E1B probe disappeared. Meanwhile, in the E1B Northern blot, due to the selection of our E1B probe (1540bp-3910bp), mRNA of the Adenovirus gene IX (3580bp-4070bp), the hexon-associated protein, was also detected. In CV890 infected Hep3B cells, gene IX expression is equivalent to that of CV802, whereas in CV890 infected HBL100, its expression was also completely shut down. This result further demonstrated that the AFP controlled E1A/E1B expression is the key for late gene expression as well as viral replication.

Results of the same samples in the Western blot also indicate that CV890 has AFP dependent expression of E1A and E1B. Under our experimental conditions, E1A expression level of CV890 in Hep3B cells is similar to that of CV802. However, when E1B 19kD protein was detected, it was found that the expression level was much lower than CV802 E1A. Previously, it has been addressed that IRES-mediated second gene has less expression (Mizuguchi et al., 2000, *Mol. Ther.* 1:376-382). Taken together, CV890 infection in permissive Hep3B cells can produce normal amounts of E1A and lesser amounts of E1B proteins capable of initiating a normal productive infection. In AFP^- cells, however, this process was attenuated due to a lack of E1A and E1B gene transcription and translation. These data demonstrated that the expression of both E1A and E1B genes are under the control of AFP TRE and the artificial E1A/E1B bicistronic cassette is functioning properly in CV890.

<u>In Vitro Replication Specificity of CV890 in Tumor Cells and Primary Cells</u>

From *in vitro* comparison of virus yield, CV890 has a better specificity profile than CV732 (CV732 is an AFP-producing, cell-specific adenovirus variant in which the E1A gene is under control of AFP-TRE). In order to gain further insights

of using CV890 in liver cancer therapy, more tumor cell lines and primary cells were tested to characterize *in vitro* virus replication. First, all cells in the study were analyzed for their AFP status by AFP immune assay. Based on AFP produced in the cells and media, all the cells were divided into three groups, high (>2.5 μg/10^6 cells/10 days), low (<0.6 μg/10^6 cells/10 days) and none (undetectable in our study) (Table 15). It was confirmed that replication of CV890 in different cell lines correlates well with the AFP status of the host cell. Among the group of liver cell lines, CV890 only replicates well in AFP^+ cells, including Hep3B, HepG2, Huh7, SNU449 and PLC/PRF/5. The amount of AFP required for the promoter activity seems very low as one of the hepatoma cell lines, SNU449, a previous reported AFP^- cell (Park et al., 1995, *Int. J. Cancer* 62:276-282), produces very low AFP (about 60 ng/10^6 cells/10 days) compared to other cells. Nevertheless, even with very low amount of AFP, SNU449 cells can still support CV890 replication to the extent comparable to cells producing significantly higher levels of AFP such as HepG2. Compared to CV802, CV890 is attenuated 5,000 to 100,000 fold in cells that do not produce AFP, including the hepatoma cell Sk-Hepl and Chang liver cell, other tumor cells and primary cells. Taken together the results indicate that CV890 has shown a good specificity profile from a broad spectrum of tumor cells. Among them, only the AFP^+ liver cells, AFP production level from high to low, are permissive for CV890.

In another experiment, CV890 was compared to CV802 for their single step growth curves on different cells. Results demonstrated that CV890 has a similar growth kinetics to wild-type CV802 in AFP^+ cells except that virus yields are slightly lower (2-8 fold) in low AFP producing cells. In consideration of experimental error, there is no dramatic difference in the replication of CV890 and CV802 in AFP^+ hepatoma cells. However, the growth curves of CV890 in AFP^- cells showed clear attenuation. During a 5 day experiment, CV890 failed to replicate in AFP^- cells including hepatoma cell (Change liver) and primary cells (nHLFC). From all the *in vitro* virus replication studies, it is clear that replication of CV890 is under the tight control of AFP-TRE and this adenovirus variant has an excellent specificity profile of preferentially targeting AFP producing hepatocellular carcinoma cells.

***In Vivo* Specificity and Efficacy of CV890**

CV890 specificity was also evaluated in animals bearing prostate cancer LNCaP xenografts. In this *in vivo* test, nude mice with prostate xenograft were intravenously injected with either CV890 or CV787, a prostate cancer specific adenovirus variant (Yu et al., 1999, Cancer Research, 59:4200-4203). Tumor volumes were documented and indicated that only CV787 had a significant antitumor efficacy in LNCaP xenografts, while tumors in the animals treated with CV890 grew, from 400 mm^3 to approximately 1200 mm^3 in six weeks, similar to the group treated with vehicle. This study indicates that CV890 does not attack LNCaP xenograft and keeps the good specificity profile under *in vivo* conditions.

To evaluate *in vivo* antitumor efficacy of CV890, different studies were carried out in the nude mouse model harboring human hepatoma xenografts. First, BALB/c nu/nu mice with HepG2 or Hep3B xenografts were established, animals were further challenged with single dose or multiple doses of CV890 into the tumor mass (intratumoral administration, IT) or via their tail vein (intravenous administration, IV). Tumor volume and the level of serum AFP were monitored weekly after the start of treatment, and hence the efficacy of the treatment was determined. The *in vitro* cytotoxicity study has demonstrated that CV890 has a better cytolytic effect than CV732. In order to further examine their antitumor activity, we first conducted animal study to compare CV890 to CV732. Animals harboring 300 mm^3 Hep3B xenograft were grouped (n=6) and injected with vehicle alone (control group), CV890 ($1x10^{11}$ particles/dose, CV890 group), or CV732 ($1x10^{11}$ particles/dose, CV732 group). The Hep3B xenograft is a very aggressive tumor model and tumors grow very fast. Most animals can not survive long because of excessive tumor burden. During a six week study, single intravenous administration of CV890 have shown significant tumor growth inhibition, whereas control mice had over 10 fold tumor growth at week 5. In the group treated with CV732, single dose IV injection also reduced the tumor growth as compared to control group, however, it was much less effective compared to CV890. For example, the average tumor volume of the CV890 treated group dropped from 312 mm^3 to 219 mm^3, while tumor volume increased from 308 mm^3 to 1542 mm^3 5 weeks after treatment in control. Both control group and the CV732 group were terminated at week 5 because excessive tumor size. Previously, CV732 has been

demonstrated to restrict the hepatoma tumor from growth after 5 doses of intravenous administration. Similar efficacy can be achieved with just a single intravenous administration of CV890, indicating that under *in vivo* conditions, CV890 has better efficacy than CV732 in hepatoma xenografts. In this experiment, 4 out of five CV890 treated mice were tumor free three weeks after treatment. However, in CV732 group, xenografts in two mice stopped growing but none of treated animals were tumor free through the six-week experiment. There was no tumor reduction in this group or the control group of animals. By statistical analysis, the differences in mean relative tumor volumes and serum AFP concentrations between CV890 treated and CV732 treated or vehicle treated tumors are significant ($p<0.01$)). Taken together, these studies suggest that CV890 has a significant antitumor activity and its oncolytic efficacy is better than CV732, an adenovirus variant similar to AvE1a04I, in which the AFP TRE was applied to control E1A alone (Hallenback et al, 1999, Hum. Gene. Ther., 10:1721-1733).

Synergistic Antitumor Efficacy of CV890 in Combination with Chemotherapeutic Agents

In this example, different chemotherapeutic agents were tested in combination with CV890 for their *in vitro* killing effect in Hep3B or HepG2 cells. Drug concentrations were optimized to the extent that they would not generate extensive cytotoxic effect on their own. Under such conditions, some agents had shown higher cell killing effect in combination with CV890. Among them, doxorubicin, a drug currently used in treatment of HCC showed synergistic cytotoxicity with CV890. In experiments using doxorubicin together with CV890 on Hep3B cells, doxorubicin at 10ng/ml did not generate cytotoxicity, whereas CV890 at an MOI of 0.01 (pfu/cell) only had about 35% of cell killed at day 9. However, when both were applied together, 90% cells were killed 9 days after treatment. In order to determine the potential synergistic effect from the combination treatment, the MTT cell viability data were subjected to further statistical analysis. Figure 38 shows a representative IC_{50} isobologram of doxorubicin and CV890 on Hep3B cells at day 5. First, the dose-response curves of doxorubicin alone or CV890 alone were made. Based on the original theory of Steel and Peckham (1993) and method by Aoe et al. (1999), three isoeffect curves (mode I and mode 2a, 2b) were constructed. From this isobologram, several data points were in the synergy or additive area,

indicating that combination of CV890 and doxorubicin provides synergistic effect on killing of Hep3B cells.

Although CV890 alone has good antitumor activity, we applied combination therapy with doxorubicin for *in vivo* evaluation of synergy. Animals harboring 300 mm^3 Hep3B xenografts were grouped (n=6) and injected with vehicle alone (control group), CV890 alone ($1x10^{11}$ particles/dose, CV890 group), doxorubicin alone (10mg/kg, doxorubicin group), or CV890 in combination with doxorubicin (combination group). Figure 38 shows weekly change of the relative tumor size normalized to 100% at day 1. In this experiment, by week six, all animals in the control group had excessive tumor which has increased by 700% of baseline, whereas in CV890 group and combination group, animals had either tumor free or tumor reduction. Of the eight Hep3B xenografts, treated with CV890, three animals (37.5%) had no palpable tumor at week 5, another three animals had tumor regressed by more than 60%. In combination group, four out of eight animals were tumor free from week 5, another four animals had tumor reduction about 90%. All the animals in the CV890 and combination group were alive and tumor was suppressed even ten weeks following treatment whereas the control animals were sacrificed for excessive tumor burden after week 6. Furthermore, CV890 also caused a drop in the serum AFP concentration in these mice. Statistical analysis shows that differences in mean relative tumor volumes and serum AFP concentrations between CV890 and vehicle treated group or combination and doxorubicin treated group are significant at different times ($p<0.005$).

The strong efficacy in the combination treatment shows that single IV injection of CV890 in combination of doxorubicin can eradicate aggressive Hep3B xenografts in most of the animals.

Table 15. AFP production in different tumor cells

AFP

CELLS	(ng/10^6cells/10days)	
Hep3B	2645	High
HepG2	3140	
HuH7	4585	
SNU449	60	Low
PLC/PRF/5	600	
Chang	0	None
SK-Hep1	0	
HBL100	0	
PA-1	0	
LoVo	0	

Example 21: CV706 in combination with Irradiation produces synergy

Materials and Methods

Cell culture and virus

The human LNCaP (prostate carcinoma), OVCAR-3 (ovary carcinoma) and HBL-100 (breast epithelia) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The human embryonic kidney cell line, 293, which expresses the Adenoviral E1A and E1B gene products, was purchased from Microbix Biosystem, Inc. (Toronto, Canada). Cells were maintained at 37°C with 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Hyclone, Utah), 100 units/ml penicillin and 100 µg/ml of streptomycin (Life Technologies, Gaithersburg, MD).

CV706 is a prostate-specific replication competent Adenovirus variant. One prostate-specific transcription response element (TRE), the human prostate-specific antigen promoter and enhancer (PSE), was inserted upstream of the E1A encoding region in the viral genome (Rodriguez et al., 1997, Cancer Research, 57: 2559-2563).

Similarly, CV787 is also a prostate-specific replication competent Adenovirus variant, which contain two prostate-specific TREs, the probasin promoter and PSE, inserted upstream of the E1A and E1B encoding regions in the viral genome, respectively (Yu et al., 1999, *supra*). Both CV706 and CV787 are currently in clinical trials for organ-confined prostate cancer and metastatic hormone refractory prostate cancer (DeWeese et al., 2001).

Cell Viability and Irradiation

MTT assays were performed to measure cell viability as described by (Yu et al, 1999, *supra*). Briefly, HBL-100, OVCAR-3 and LNCaP cells ($2x10^4$ cells/well, 96 well plate) were either infected with CV706 or CV787 at various MOI (from 0.0001 to 1) or treated with irradiation at the indicated dosages. Cells were incubated in growth medium for 24 hr to allow for viral replication. After 24 hr, cells were exposed to a single dose of γ-irradiation (0 ~ 40 Gy) (Mark 1 Research Irradiator Model #1608A, Caesium 137 source). Cell viability was measured at the times indicated by removing the media and replacing it with 50 µl of 1mg/ml solution of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) (Sigma, St. Louis, MO) and incubating for 3 hrs at 37°C. After removing the MIT solution, the crystals remaining in the wells were solubilized by the addition of 50 µl of isopropanol and placed in a 30°C incubator for 30 min for the crystals to dissolve. Plates were vibrated for 10 sec prior to reading. The absorbency was determined on a microplate reader (Molecular Dynamics) at 560 nm (test wavelength) and 690 nm (reference wavelength). At least, 8 replica samples were taken for each time point and the percentage of surviving cells was estimated by dividing the OD_{560} - OD_{690} of virus infected cells by the OD_{560} - OD_{690} of mock infected cells.

Statistical Analysis

The dose-response interactions between CV706 and irradiation at the point of IC_{50} were evaluated by the isobologram method of Steel and Peckham as modified by Aoe et al. (Aoe et al. 1999, Anticancer Res. 19:291-299). The IC_{50} defined as the concentration of drug that produced 50% cell growth inhibition, i.e. 50% reduction in

absorbance. Isobolograms (three isoeffect curves, model 1 and model 2) were computed as described previously (Yu et al., 2001). Fractional tumor volume (FTV) relative to untreated controls was determined based on the method described previously (Yokoyama et al., 2000; Yu et al., 2001, Cancer Research).

On Step Growth Curve and Virus Yield

One-step growth curve of CV706 in the presence and absence of irradiation were performed in LNCaP cells to determine burst size. Monolayers of LNCaP cells were infected with CV706 at MOIs 0.01, 0.1 and 1. After 24 hour incubation at 37°C with 5% CO_2, cells were exposed to a single dose of γ-irradiation at 10 Gy. At the indicated times thereafter, duplicate cell samples were harvested and lysed by three cycles of freeze-thawing. Virus yield was determined by plaque assay as described in (Yu et al., 1999, Cancer Research, 59:1498-1504).

***In Vivo* Antitumor Efficacy**

Six to eight week old athymic Balb/c nu/nu mice were obtained from Simonson Laboratories (Gilroy, CA) and acclimatized to laboratory conditions one week prior to tumor implantation. Xenografts were established either by injecting 1 x 10^6 LNCaP cells subcutaneously near the small of the back suspended in 100 µl of RPMI 1640 and 100 µl of matrigel (back tumor) or by injecting cells into the right gastrocnemius muscle (i.m.) (leg tumor). When tumors reached between 300 mm^3 and 500 mm^3, mice were randomized into groups of four. The first group received CV706 at day 0 via intratumoral (i.t.) administration. CV706 was diluted by PBS containing 10% glycerol and injected into tumor as 0.4 µl of diluted virus ($1x10^7$ particles) per mm^3 of tumor using a 28-gauge needle. The second group was given irradiation only. For irradiation mice were immobilized in lucite chambers and their whole body was shielded with lead except for the tumor bearing sites on their back or tumor-bearing hind leg. This tumor-bearing site in back or leg was irradiated with a Mark 1 Research Irradiator (Model #1 608A, J.H. Shepherd Associates) at various doses (0, 5, 10 and 20 Gy) 1 day after CV706 injection or mock injection. The third group was given CV706 (i.t.) at day 0 and irradiated at the same doses at day 1. As a control, a fourth group was treated with virus dilution buffer (i.e. control) i.t. at day

0. Tumors were measured weekly in two dimensions by external caliper and volume for back tumors was estimated by the formula [length (mm) x width $(mm)^{2]}/2$ (Yu et al., 1999b). Volumes of i.m. leg tumors were determined using the following formula (Alfieri and Hahn, 1978, Cancer Research, 38:3006-3011): volume (cm^3) = $d'3-(0.6)^2d'$, where d' is the average diameter of the tumor-bearing leg (cm), and the product $(0.6)^2d'$ is the correction factor for normal leg volume. Animals were humanely killed when their tumor burden became excessive. The difference in relative tumor volumes between treatment groups was compared for statistical significance using the type 2 (two-sample equal variance), two-tailed, *t*-test. Blood samples were collected at various time points by retro-orbital bleed for determining prostate-specific antigen. Federal and institutional guidelines for animal care were followed.

Histochemistry Analysis

Four groups of mice (n=6) were treated with vehicle, CV706 ($1x10^7$ particles per mm^3 of tumor), irradiation (10 Gy) or a combination of CV706 and irradiation. Half the animals were sacrificed on day 7 and the other half on day 14. The tumor samples were embedded in paraffin blocks and 4-μm sections were cut and stained with Hematoxylin and Eosin (H&E). Histology methods for detecting Adenovirus antigens were as described (Yu et al., 1999, Cancer Research, 59:4200-4203). The necrotic cells were scored on coded slides at light microscopy at x 400 magnification. The number of necrosis was based on scoring 500 points per section as either necrotic or nonnecrotic. The average necrosis score was calculated based on counting in 10 fields distributed evenly across the area of tumor section. The light-microscopic features used to identify necrosis included cell size, indistinct cell border, eosinophilic cytoplasm, loss or condensation of the nucleus, and associated inflammation (Milross et al., 2000). To assess the effect of CV706, irradiation or the combination treatment on tumor vascularization, the number of blood vessels was counted at a magnification of x 400 and the average blood vessels were calculated from 10 fields distributed evenly across the area of whole tumor section. Apoptotic cells were detected using TUNEL assay (Roche Molecular Biochemicals, Indianapolis, IN) as suggested by the manufacturer. The morphological features

used to identify apoptosis in the tumor sections have been previously described, associated with positive terminal deoxynucleotidyl transferase-mediated nick end labeling staining (Milross, et al., 2000). The apoptotic cells were scored on coded slides at x 400 magnification and average score of apoptotic cells was calculated from 10 fields of nonnecrotic areas selected randomly across each tumor section.

RESULTS

CV706 in Combination with Irradiation Produce Synergistic Cytotoxicity in Prostate Carcinoma LNCaP Cells

To study the potential interaction between a prostate-specific Adenovirus variant CV706 and radiation *in vitro*, the effectiveness of combined treatment of several combinations of CV706 and irradiation at various doses was evaluated in the PSA-producing prostate carcinoma LNCaP cell line. LNCaP cells were either mock-infected, or infected with CV706. One day later, cells received a single dose of γ-irradiation (0, 5Gy, 10Gy and 20Gy) and the cell viability was then determined at various time points by the MTT assay. Several viral MOIs and radiation doses were tested to determine the dose-response curves in LNCaP cells, such that the selected dose shows greater combined efficacy with radiation or virus, but minimal cell killing when treated with the same dose of virus alone or radiation alone. Infecting LNCaP cells with CV706 at an MOI of 0.01 resulting in 80% cell survival 5 days after infection, while irradiation at a dose of 10 Gy resulted in 78% survival 5 days after treatment. However, when CV787 and radiation were combined at these doses, cell survival dropped to 20% 5 days after treatment. Cell viability dropped further to 8% 9 days after combination treatment, while cells treated with virus at MOI 0.01 alone or radiation 10 Gy alone retained 70% or 60% cell viability, respectively.

Isobolograms were generated from the models to determine the presence of synergy, additivity, or antagonism between CV706 and irradiation. The results indicate that sequential exposure to CV706 followed by irradiation produced synergistic cytotoxicity. The enhanced cytotoxicity was also observed in LNCaP cells when CV787, a second prostate-specific Adenovirus variant, was combined with radiation Taken together, our *in vitro* data demonstrate that prostate-specific

Adenovirus variants in combination with irradiation produce synergistic cell cytotoxicity in prostate carcinoma LNCaP cells.

Irradiation Increases CV706 Burst Size in LNCaP Cells

Irradiation kills mammalian cells in the reproductive (also known as clonogenic) death pathway. DNA is the target, and double-stranded breaks in the DNA are regarded as the specific lesions that initiate this lethal response. Most radiation induced DNA double-stranded breaks are rapidly repaired by constitutively expressed DNA repair mechanisms. Residual unrepaired or misrepaired breaks lead to genetic instability and to increased frequency of mutations and chromosomal aberrations (Garzotto et al., 1999). Because of its small target size, the adenoviral genome (36kb) is far less likely to sustain radiation-induced damage as it is 10^5-fold smaller than that of human cells ($3x10^6$ kb).

To examine the effect of irradiation on virus replication, we performed a one-step growth curve. LNCaP cells were infected with CV706 at an MOI of 0.1 for 24 hrs, followed by irradiation at a dose of 10 Gy. Cells were harvested at various times post-infection and the number of infectious virus particles was determined on 293 cells by standard plaque assay (Yu et al., 1999, supra). Although the initial rate of increase of CV706 in cells treated with CV706 and irradiation was similar to that of cells treated with CV706 alone, cells treated with CV706 and irradiation produced a larger burst size than CV706 alone. For example, cells treated with CV706 and irradiation produced 8,000 PFU per cell 9 days post-infection, while the cells infected with CV706 alone generated about 500 PFU per cell 9 days after virus infection. A bigger virus burst size was also observed in the combination treatment of irradiation and CV706 at MOIs 0.01 or 1. Cells treated with CV706 at MOI of 0.01, and 1 produced 15 and 3500 PFU per cell, whereas cells treated with CV706 at MOI of 0.01 and 1 combined with irradiation, produced 4750, and 8700 PFU per cell respectively, at 9 days after virus infection. Thus, irradiation does not inhibit CV706 replication, but significantly increases virus propagation.

Cytotoxicity of CV706 in Combination with Irradiation Remains to Be Specific to Prostate Cancer Cells

In order to evaluate whether the addition of radiation could change the specificity of CV706's cytotoxic activity, we assess the specificity of the combination treatment of CV706 and radiation by measuring viability of various infected cell lines using the MTT assay. LNCaP, HBL-100 and OVCAR-3 cells were infected with CV706 at an MOI of 0.01 for 24 hrs, followed by a single dose of radiation at 10 Gy. The percentage of cell viability versus time post treatment was plotted. The combination of CV706 and radiation was toxic to LNCaP cells, but not to HBL-100 and OVCAR-3 cells. There were few surviving LNCaP cells 9 days after infection. In contrast, the viability of HBL-100 and OVCAR-3 cells treated with CV706 and radiation was more than 90% throughout the course of the experiment, similar to that of cells treated with radiation alone. This data suggests that combination with irradiation does not alter CV706's specificity.

Synergistic Efficacy of CV706 in Combination with Irradiation *In Vivo*

The *in vivo* antitumor efficacy of CV706 in combination with irradiation was assessed in the LNCaP mouse xenograft model. We have shown previously that a single intratumoral administration of CV706 at $5x10^8$ particles per mm^3 of tumor can eliminate subcutaneous xenograft tumors in 6 weeks (Rodriguez et al., 1997,*supra*) Established human prostate cancer xenografts (LNCaP cells) were treated with either vehicle, CV706 ($1x10^7$ particles/mm^3), irradiation (10 Gy), or both CV706 and irradiation. For the combination treatment, animals were intratumorally injected with either CV706 or vehicle, and 24 hours later, animals received a single dose of irradiation. In this study, a single dose of 10Gy was used because it caused a tumor growth delay in a previous pilot study. The dose of $1x10^7$ particles per mm^3 of tumor was selected based on our previous studies on its antitumor efficacy (Yu et al., 1999, supra.

The tumor volume data shows that there was a significant decrease in tumor volume between control and all treatment groups. In all cases although single doses of CV706 or irradiation were effective in producing tumor growth inhibition, the combination of the two showed significant tumor regression. For example, tumor

volume of the group treated with irradiation (10 Gy) was 119.76% of baseline 6 weeks after treatment, while the tumor volume of the group treated with CV706 was 97.39% of baseline 6 weeks after administration. However, when CV706 was combined with irradiation at similar doses, a statistically significant drop in the relative tumor volume (4% of baseline) was observed ($p<0.01$). Additionally, relative PSA level in serum of mice was also monitored for anti-tumor efficacy. Relative PSA level in mice increased to 370% of baseline 6 weeks after receiving vehicle treatment, increased to 139% after receiving irradiation alone, reduced to 84% of baseline after being treated with CV706 alone, whereas the PSA levels in mice treated with CV706 and irradiation decreased to less than 1% of their starting values within 6 weeks.

After 7 days, combination treatment showed more than additive effect on tumor growth inhibition at all the time points studied. On day 21, there was more than 2-fold improvement in anti-tumor activity in the combination group when compared with the expected additive effect. At this time point, both CV706 and irradiation (10 Gy) per se inhibited tumor growth by 26% and 34%, respectively (fractional tumor volume, 0.7419 mm^3 and 0.6645 mm^3, respectively) when compared with the control group. This anti-tumor activity further improved with time. On day 42, the group treated with the combination of CV706 and irradiation showed a 6.69-fold higher inhibition of tumor growth over the expected fractional tumor volume. These observation further strengthen the idea of synergy between CV706 and irradiation in the eradication of LNCaP xenografts.

Enhanced antitumor efficacy was also observed in the animal model in which the prostate cancer tumors are implanted in hind limb of mice. In this study, tumors were produced by inoculation of $1x10^6$ cells into limb muscle. Those tumors which were attained a volume of 200 mm^3 to 300 mm^3 were randomized into four groups and treated as described above for back tumors. As before the weekly tumor volume measurements showed that combination treatment of CV706 and irradiation led to significant antitumor activity in comparison to either CV706 or irradiation. For example, tumor volume of the group treated with irradiation (20 Gy) was 70% of baseline 4 weeks after treatment, while the tumor volume of the group treated with CV706 ($5x10^7$ particle per mm^3 of tumor) was 75% of baseline 4 weeks after

administration. However, when CV706 was combined with irradiation at these dose levels, the tumor volume dropped to 8% of baseline.

A series of experiments were then designed to examine the effects of various factors, including the sequencing of the agents, timing of irradiation following virus administration and irradiation fractionation. The effect of order of administration for the tested agents was examined in an *in vivo* study using back tumor xenograft model. LNCaP xenografts were irradiated 24 hr before or after CV706 administration. Weekly measured tumor volume indicated that treatment with CV706 prior to irradiation was significantly superior to irradiation followed by CV706.

The second study was designed to evaluate the timing of irradiation following virus administration. Tumors were treated with CV706 at day 0 and followed by irradiation at various periods of time. The results of average tumor volume indicated that similar antitumor efficacy was achieved when tumors treated with CV706 at day 0 following by irradiation 1 day or 4 days after virus administration, both eliminated tumors within 6 weeks after treatment. However, the antitumor activity was decreased when the tumors were treated with irradiation 7 days after CV706 administration.

The third study was designed to assess the effect of radiation fractionation on antitumor efficacy. Animals with human prostate cancer tumors on their backs were randomized into five groups. Two of which were treated with either CV706 at day 0 followed by a single dose of radiation at 10 Gy on day 1, or CV706 at day 0 followed with four fractional doses of radiation at 2.5 Gy on day 1, 2, 6 and 8. Weekly measured tumor volume data indicated that both treatments eliminated the pre-existing tumors 6 weeks after treatment and produced an synergistic antitumor activity when compared to either agent alone. However, no significant difference in antitumor efficacy was observed between these two combination groups as long as the total doses of irradiation was the same.

Synergistic antitumor efficacy of CV706 in combination irradiation was further documented by tumor histological analysis. First of all, more necrotic cells were observed in the tumors treated with CV706 plus irradiation compared with

either agent alone. The amount of necrosis in tumors treated with CV706 alone was higher than control tumor or tumor treated with radiation. Evidence of necrosis and multifocal inflammation was observed in a small portion of tumors treated with radiation. In the tumor treated with both the virus and radiation, a few virus-infected cells were detected. Most of the cells in the sections were empty and virtually devoid of cellular content. Significantly increases in the extent of necrosis was a dominant histological feature, which makes up about 95% of the tumor mass in this treatment group. The average necrosis scores in a x 400 magnification for the tumors treated with vehicle, radiation, CV706 and both were 5.4±2.17, 67±48.24, 258.2± 80.76 and 461.6±37.87, respectively. The presence of mass necrosis in the tumors treated with CV706 or CV706 plus radiation suggests that the induction of necrosis greatly attributes CV706 or CV706 plus radiation's anti-tumor efficacy *in vivo*. Student T test showed that tumor cell necrosis caused by CV706 in combination with radiation was significantly greater than by CV706 ($p<0.03$) and irradiation perse ($p<0.0001$). This observation is in agreement with the number of apoptotic cells observed in the treated tumors. The number of apoptotic cells, detected using TUNEL assay (Milross et al., 2000) in the tumors treated with CV706 and irradiation is 16-fold higher than vehicle, 8.8-fold higher than irradiation and 3.2-fold higher than CV706.

Secondly, a significant reduction in blood vessel numbers was observed in the tumors treated with CV706 in combination with irradiation. Average number of blood vessel observed at a magnification of 400x in tumors treated with vehicle, CV706, radiation or the combination of CV706 and radiation were 87.5±6.3, 27.5±8.9, 58.5±3.1 and 4.5±1.9, respectively. Significantly reduced numbers of blood vessels in the tumors treated with combination in comparison to CV706 alone or irradiation alone ($p<0.01$) suggest that the reduction of tumor vascularization may contribute to enhanced tumor regression. It is unclear at this time as to the precise mechanism by which this reduction in blood vessel number is achieved. The possibility for such an eventuality through direct damage of endothelial cells or indirectly through the destruction of tumor vasculature by extensive necrosis seems highly possible. CD31 is expressed constitutively on the surface of adult and embryonic endothelial cells and has been used as a marker to detect angiogenesis

(Giatromanolaki et al., 1997, Clin. Can. Res. 3 (12pt 1): 2485-92). Immunohistochemical staining was performed to examine the effect of treatment on tumor angigenesis by using monoclonal antibody against CD31 (Horak et al., 1992). Tumors treated with CV706 followed by irradiation showed a significantly lower level of CD31 positive vessel when compared to radiation ($p=0.003$) or CV706 alone ($p=0.03$). When compared to untreated mice, CV706/radiation treated mice exhibited significantly lower (4-fold) CD31 positive blood vessel counts ($p<0.0001$), whereas, radiation treated or CV706 treated mice displayed 1.6-fold ($p=0.03$) or 2.1-fold ($p=0.004$) lower CD31 positive blood vessel counts. These observations suggest that CV706 in combination with radiation may be inhibiting tumor angiogenesis to a significant extent.

Finally, treatment employing the combination seems to have a beneficial effect on the general health of the treated animals in comparison to the individual treatment. The quality of life of the treated animals seems to be greatly improved as evidenced by the general appearance and significant gain in the body weight. Indeed, animals treated with both CV706 and irradiation gain 38% more weight than untreated control animals, 22% more than CV706 treated animals and 25% more weight than irradiation treated animals. The combination treatment seems to protect the animals from the transient weight loss observed in the case of animals treated with irradiation alone.